

Review Article

***Aggregatibacter actinomycetemcomitans*: The virulence factors and relation to persistence biofilm formation**

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Corresponding author: **Norzawani Jaffar**. Email: zawanijaffar@unisza.edu.my**ABSTRACT**

Periodontitis is an infectious and inflammatory condition that is associated with subgingival biofilms in tooth-supporting tissues. Among the several hundred isolated organisms in the oral cavity, one of the most isolated bacteria from infected periodontal pockets are *Aggregatibacter actinomycetemcomitans*. It is a Gram-negative, facultative anaerobic bacillus that causes juvenile (localized aggressive periodontitis) and adolescent periodontal diseases. The development of biofilms is an essential factor in pathogenesis for *A. actinomycetemcomitans*. The early attachment of *A. actinomycetemcomitans* to abiotic surfaces relies on its protein-like fimbriae. This organism's ability to form tenacious biofilms can determine its survival and progression. *A. actinomycetemcomitans*, a pathogen not solely in periodontal but also involve in some systemic infections. This species has several virulence factors and genes that contribute to its oral cavity survival and, worst of all, cause bone resorption and tooth loss. Genetic diversity between the different *A. actinomycetemcomitans* isolates are great, and their ability to express and release virulence factors varies. In this review article, we discuss about the potential virulence factors and candidates genes for *A. actinomycetemcomitans* and their roles within periodontal disease by revealing their functional biology in facilitating attachment to oral surfaces, hindering protection of the host and causing inflammation and degradation of tissue.

Keywords: Periodontal disease; *Aggregatibacter actinomycetemcomitans*; biofilm; virulence factors; virulence genes

INTRODUCTION

Periodontal disease is an inflammatory state of the teeth affecting gum and bone support. Global Burden of Disease Study (1) reported that half of the world's population (3.58 billion people) were affected by oral diseases, with dental caries being identified as the most prevalent condition. In 2016, severe periodontal disease was also the 11th most prevalent disease worldwide. Among the several hundred species isolated in the oral cavity, *Aggregatibacter actinomycetemcomitans* is one of the most commonly isolated bacteria in infected periodontal pockets. *A. Actinomycetemcomitans* is a Gram-negative, non-motile coccobacillus anaerobic bacterium that colonizes the oral cavity of humans and is often correlated with aggressive periodontitis (2). This bacterium is a member of the Pasteurellaceae and HACEK group among the pathogen community. *A. Actinomycetemcomitans* was first reported by Klinger in 1912, who named it as *Bacterium actinomycetemcomitans* and later was modified by Lieske into *Bacterium comitans* in 1921, and finally it was renamed as *Actinobacillus actinomycetemcomitans* by Topley and Wilson in 1929 (3). In 2006, it was reclassified as *Aggregatibacter actinomycetemcomitans* by Nørskov-Lauritsen and Kilian (4). The formation of biofilms represents significant virulence and

pathogenesis factor for *A. actinomycetemcomitans*. The extracellular polymer matrix containing polysaccharides, proteins, and extracellular DNA (eDNA) help microbial cells in biofilms to be retained. This biological activity is exerted by the functional virulence factors and genes in *A. actinomycetemcomitans* to promote settlement and invading the epithelial cell and thus forming a matured and tenacious biofilm on it. So, in this review, the background details and several virulence factors of *A. actinomycetemcomitans* and their functional genes involved will be elaborated.

Periodontal Disease

Periodontal disease is a common type of disease involving inflammatory tissue-supporting conditions, for example, oral microbial, dental plaque and alveolar bone. Periodontal disease occurs when oral bacterial biofilm disturbs the connective tissues surrounding and supporting the tooth. The action induces an inflammatory response that contributes to the breakdown of the binding tissue attachment to teeth, alveolar bone, and eventually causes tooth loss (5). Gum bleeding, swollen gums, irritation, and sometimes bad breath can be seen as the symptom. Loss of gum attachment to the tooth in its more severe form causes "pockets" and loosening of the teeth. Noteworthy, poor oral hygiene and tobacco use are the main causes of periodontal disease as well as granulomatous disorders, hematological,

dermatological, neoplastic, and immunosuppression. Genetic, environmental factors are a number of other factors that contribute to the development of periodontal disease, including stress, age, medication, diabetes, and smoking.

Several studies have shown that through general inflammatory effects or direct metabolism of chemical mutagenic agents, certain oral microbiomes could induce systemic or chronic disorders. According to Haubek (6), widespread periodontal disease has been linked with diabetes, adverse outcomes of pregnancy, pulmonary disease, and stroke. Moreover, periodontal can cause systemic severe conditions like pneumonia (7) and cardiovascular disease (8). Additionally, severe bacterial infections could be the cause or agent of oral cancer, as a number of periodontal bacteria and large bacterial salivary counts are associated with oral squamous cell carcinoma (9).

Role of *A. actinomycetemcomitans* in periodontal disease

In 1975, *A. actinomycetemcomitans* was first reported in localized juvenile periodontitis, now known as localized aggressive periodontitis (LAP) as a potential periodontal pathogen. Most cross-sectional findings have since revealed *A. actinomycetemcomitans* in adolescents are closely associated with periodontal disease. Recent reports by Kawamoto *et al.*, have shown that in peri-implantitis and periodontitis, *A. actinomycetemcomitans* can promote osteoclast formation and bone loss by inducing the secretion of interleukin 6 (IL-6) from human gingival fibroblasts (HGFs). In addition, Interleukin 8 (10) and interferon-inducible protein-10 (11) that plays a major role in bone resorption are regulated to correspond with the level of periodontal inflammation in periodontal diseases.

Biofilm formation of *A. actinomycetemcomitans*

The initial adhesion of *A. actinomycetemcomitans* to abiotic surfaces depends on protein fimbriae (12), polysaccharides (13) and extracellular DNA (14). Bacterial biofilms are bacterial communities where microbial cells are bound to the substrate surrounded by an extracellular polymeric material matrix. Polymeric β -1,6-N-acetyl-D-glucosamine (PGA) is a significant component of the cell-surrounding matrix in these biofilms (13). In *A. actinomycetemcomitans*, PGA development is regulated by enzymes encoded in the *pgaABCD* operon, and PGA biosynthesis involves the expression of all four genes (14). This lifestyle of biofilm is the cause of most infections involving bacteria. In addition, bacterial cells in biofilms can effectively evade the host immune system, possibly via inhibition of engulfment of biofilm cells by phagocytes. This may be due to biofilm matrix polymers, which are of low

immunogenicity and have the ability to mechanically shield the surface-exposed epitopes of bacterial cells in biofilms from being recognized by the host immune system.

Compared with their planktonic stage, bacteria in biofilms are distinguished by intense antimicrobial resistance. In addition, biofilm cells are actively monitoring their surroundings and adapting their properties dynamically to the prevailing environment. Therefore, when needed, bacteria will express their necessary genes. Expression of bacterial virulence genes, for example, is regulated by sensing changes in the levels of certain compounds, such as bacterial signaling molecules, host compounds, toxic oxidants, and iron (15). Particularly, the bacteria living in biofilms suggested been using cytokines to modulate gene expression and biofilm formation. Such a mechanism cannot be observed in the planktonic form of bacteria, which indicates that the genes react to cytokines are regulated in biofilm form (16). This mechanism is appropriate for the progressiveness of periodontal disease as an abundance of bacterial biofilms found in the oral cavity.

***A. actinomycetemcomitans* serotypes and pathogenicity**

The serotypes of *A. actinomycetemcomitans* have already been investigated. There are several serotypes discovered. Seven serotypes have been identified so far, such as serotypes a, b, c, d, e, f, and g, leaving 3-8 % non-serotypeable of clinical isolates (17) with more than one serotype in an individual's mouth. According to Umeda *et al.*, (18), genome analysis of *A. actinomycetemcomitans* could be classified into two major groups of serotype-specific polysaccharide antigens: serotypes: a, d, e, and f, and serotypes b and c, to further explain their virulence variations. Among these serotypes, the most prevalent in the oral cavity are serotypes a, b, and c (3). Furthermore, serotype b is often associated with aggressive periodontitis in young people, but the virulence and toxicity were not found to be strictly following the serotype pattern (19). Each *A. actinomycetemcomitans* serotype comprises a unique phenotypic and genotypic feature, but little is known about core gene expression and its effects on the virulence potential (20).

Virulence factors of *A. actinomycetemcomitans*

It is indicated that *A. actinomycetemcomitans* has many virulence factors contributing to their survival in the oral cavity and allow them to combat the defense mechanism of the host. *A. Actinomycetemcomitans* expression of virulence factors can be divided into three groups based on Umeda *et al.*, (Table 1; 18) with its virulence genes (Table 2).

Table 1: Virulence factors of *A. actinomycetemcomitans*.

Groups	Virulence Factors
1. Facilitating attachment to oral surfaces.	- adhesions - invasions - antibiotic resistance
2. Hindering the protection of the host.	- leukotoxins - cytolethal distending toxin (CDT)
3. Causing inflammation and degradation of tissue.	- lipopolysaccharide - heat-shock protein (HSP)

1. Facilitating attachment to oral surfaces

A. Adhesions

Bacterial adherence is the first step in invading cells. Adhesins are components of the bacterial cell surface that mediate bacterial adhesion to surfaces of eukaryotic cells by binding to eukaryotic surface receptors. Fimbriae helps *A. actinomycetemcomitans* to firmly attach to surfaces such as teeth and epithelial subgingival crevicular cells to form a dense biofilm (21). The first described *A. actinomycetemcomitans* protein found to be involved in essential adherence of the organism to epithelial cells was the autotransporter adhesin protein (Aae). A study carried out by Rose *et al.*, exhibited the *aae* gene deletion from two different *A. actinomycetemcomitans* strains that was capable to minimize the ability of mutant strains to bind to epithelial cells as relative to wild species (WT). The outer membrane protein 100 (*Omp100*) of *A. actinomycetemcomitans* 's is referred to as surface-expressed for adhesin. The deletion of *omp100* gene from *A. actinomycetemcomitans* Y4 strain resulted in decreased mutant adhesion and efficacy of invasion (22). In contrast, *A. actinomycetemcomitans* *EmaA* directly binds to collagen and fibronectin (23). The elimination of this bacterial adhesin decreases the fivefold binding of *A. actinomycetemcomitans* knockout strain to the exposed collagen of the rabbit cardiac valve tissue relative to a wild bacterium (24).

B. Invasions

Following attachment, bacteria can invade the cells, which induce multiple effectors such as microfilament and microtubule (25). *A. actinomycetemcomitans* invasion is a rapid process involving opening of the cell surface. These invasions happen by indentations on the cell surface as well as in membrane ruffles (3). The cells of *A. actinomycetemcomitans* bind through adhesins to surface receptors on the gingival cells-transferring receptor. The epithelial cell membrane has been wrecked and damaged, causing the bacteria's invaginations and internalized in a membrane vesicle (26). When infiltrated into the cells, *A. actinomycetemcomitans* can avoid immune attack by

controlling genes and proteins and live in the cytoplasm.

C. Antibiotic resistance

Antibiotic resistance is a crucial global issue. The evolution of microorganism resistance toward antibiotics lead to misuse and overuse of antibiotic administration resulting in elevating antibiotic concentration in the surrounding environment (27). When the released antibiotic leads to pathogenic antibiotic-resistance bacteria into the area, it will become worse. Tetracycline resistance gene, for example, was reported in water samples collected from wastewater treatment plants near U.S. swine production facilities (28). Following this, genes of antibiotic resistance will be spreading in the exposed microorganism. Such data suggest that *A. actinomycetemcomitans* resistant to antibiotics are on the rise and likely to be responsible for potential delays in care. This problem intrigues researchers to analyze and test other alternatives against oral pathogens.

2. Hindering the protection of the host

A. Leukotoxins

A. actinomycetemcomitans 's leukotoxin is one of the main virulence factors that can kill immune tissues in the host. It is a large protein-forming pore toxin that is secreted from the *A. actinomycetemcomitans* cell membrane. This belongs to the family of bacterial cytolysins repeats-in-toxin (RTX) (29). The toxin operon consists of four coding genes such as *ltxA*, *ltxB*, *ltxC*, and *ltxD*, and the promoter is upstream of these genes (30). Type I secretion process takes the toxin to the outer bacterial membrane (31). The outer membrane structure is affected by inner membrane protein, MorC for efficient transfer of the toxin (32). Leukotoxin is cell-specific as well as species-specific. The toxin binds to neutrophils, monocytes and a subset of lymphocytes and forms openings in the membranes of these target cells, destroying their ability to maintain osmotic homeostasis, leading to cell death (3).

B. Cytolethal distending toxin (CDT)

CDT is a protein with an immunosuppressive role in the cell cycle. The toxin is either secreted freely or associated with the membrane of the producing bacteria. Strains of *A. actinomycetemcomitans* commonly associated with localized aggressive periodontitis (LAP) possessing *cdt* operon (33). This toxin comprises three subunits: subunit B (*cdtB*) is the active toxic subunit; subunits *cdtA* and *cdtC* are the target cell binding subunits; while *cdtC* supports subunit B delivery to cells. Phosphatidylinositol-3,4,5-triphosphate phosphatase activity (PIP3 phosphatase) is the function of the active subunit, *cdtB* (34) and exhibits DNase I activity (3). Cell-based *cdtB* targets are cell cycle arrest in G0/G1 and

G2/M by destroying DNA (phosphatase activity of PIP3) in T cells, macrophages, Hep-2 cells, and many epithelial cells (34). In addition, CDT induces macrophage destruction by preventing phagocytic activity and altering the balance of cytokines. In this process, cytokine production levels (IL-1 β , IL-10, and IL-12) increase, nitric oxide production is modulated, and TNF- α levels do not differ throughout macrophage cells due to the presence of CDT (35).

3. Causing inflammation and degradation of tissue

A. Lipopolysaccharide

Lipopolysaccharide (LPS) is a major external membrane element of Gram-negative bacteria that stimulates innate immune cells to initiate inflammatory responses through Toll-like receptor 4 (TLR4) (36). The TLR4 stimulates two separate signaling pathways that are MyD88-dependent pathway (NF- κ B activation) and MyD88-independent pathway. *A. actinomycetemcomitans* lipopolysaccharide induces pro-inflammatory mediators like IL-1 β , -6, -8, and TNF- α (37) and is closely linked to the adverse effects of pregnancy (38). The highlight of *A. actinomycetemcomitans* LPS is hematogenic dissemination and its impact on the fetoplacental unit, which causes low birth weight (LBW) associated with periodontitis (39). Research by Offenbacher *et al.*, (40) shows that *A. actinomycetemcomitans* in mothers with LBW is lower than healthy control mothers. *A. actinomycetemcomitans* LPS (AaLPS) is also

associated with the destruction of connective tissue and alveolar bone loss in periodontal diseases (41).

B. Heat Shock Proteins (HSPs)

During periodontal infections, environmental changes cause *A. actinomycetemcomitans* to synthesize heat shock proteins (HSPs) (42). HSPs are a set of highly conserved proteins expressed in response to cell stress, including nutrient deprivation, and play a critical role in protein folding, intracellular protein trafficking, and denatured protein coping (43). *A. actinomycetemcomitans* can induce GroEL (chaperonin 60; HSP60), DnaK (HSP80), and HtpG (HSP90) proteins to protect cells from lethal effects during heat stress (44).

GroEL interacts with a small protein composed of 10-kDa subunits (GroES) in *A. actinomycetemcomitans*. GroES and GroEL encoding genes were combined in the same process (42). Protein that is homologous to GroEL is osteolytic. GroEL was found to encourage the proliferation of epithelial periodontal ligament cells at concentrations ranging from 0.4 to 1.0 μ /mL. Increased apical proliferation in the junctional epithelium contributes to the widening of periodontal spaces, thereby creating a larger space for the growth of bacteria. Thus, in the periodontal pocket, GroEL contributes to the survival of *A. actinomycetemcomitans*. On the other hand, it is shown that a higher concentration of GroEL (10 μ g protein / mL) has cytotoxic effects on epithelial cells. Therefore, based on the abundance of *A. actinomycetemcomitans* in different areas of the body, the results of this protein could be both increased proliferation and cell death (45).

Table 2: Virulence factors, properties, genes, and its functions

Virulence Factor	Virulence Properties	Virulence Genes	Function	References
Facilitating attachment to oral surfaces Adhesion	- Involve contact and binding in saliva, on the tooth surface, on extracellular matrix proteins, and on epithelial cells (21).	<i>Aae</i>	- Autotransporter adhesion protein.	(21)
			- A homolog of epithelial cell adhesion.	(46)
		<i>Omp100</i>	- Adhesin that interacts with fibronectin.	(47)
		<i>EmaA</i>	- Extracellular-matrix protein adhesin-A	(23)
			- Homotrimeric adhesin that binds to collagen and fibronectin	(24)
			- Family of autotransporter adhesin	(48)
		<i>pgaA</i> ,	- PGA, biofilm part of	(13)

Invasion	- The cells of <i>A. actinomycetemcomitans</i> bind through adhesins, to surface receptors on the gingival cells-transferring receptor.	<i>pgaB</i> , <i>pgaC</i> and <i>pgaD</i>	the cell-surrounding matrix, internal adhesion feature	(13) (12) (49) (50)
		<i>flp-1</i> and <i>flp-2</i>	- Involve in the development of the major structural element of fimbriae, Flp1 protein.	
		<i>rcpA</i>	- Involve in the formation of rough-colony protein A – help fimbria attached to host cells by forming a channel	
		<i>apaH</i>	- Expression of an invasion-associated enzyme - The species increased its expression of <i>apaH</i> under anaerobic conditions. - Increased capacity to attack epithelial cells under anaerobic conditions.	
Hindering the protection of the host		<i>Omp29</i>	- Invasion of non-phagocytic cells	(30) (3)
Leukotoxin	- Proteinaceous toxin secreted from the cell membrane of <i>A. actinomycetemcomitans</i> . - The toxin attaches to neutrophils, monocytes, and a subset of lymphocytes; it forms pores in the membranes that overwhelm osmotic homeostasis, leading to cell death (3).	<i>ltxA</i> , <i>ltxB</i> , <i>ltxC</i> and <i>ltxD</i>	- <i>LtxA</i> codes the toxin's structural protein. - <i>LtxC</i> encodes post-translational toxin acylation components. - <i>LtxB</i> and <i>ltxD</i> encoding the toxin to the outer membrane.	(30) (3)
Cytolethal distending toxin (CDT)	- it is transported into the nucleus where it causes DNA damage through DNase activity resulting in apoptosis.	<i>cdt</i> – <i>cdtA</i> , <i>cdtB</i> and <i>cdtC</i>	- Macrophage disrupts by inhibiting phagocytic activity and influencing interleukin production 1-β, 6, and 8. - The active toxic subunit B (<i>cdtB</i>). - The <i>cdtA</i> and <i>cdtC</i> subunits are the	(35) (34)

<p>Causing inflammation and degradation of tissue</p> <p>Heat-shock proteins (HSPs)</p>	<p>- conserved proteins expressed in response to cell stress and play a critical role in protein folding, intracellular protein trafficking and denatured protein coping (43).</p>	<p>GroES and GroEL</p>	<p>subunits that bind target cells.</p> <ul style="list-style-type: none"> - CdtC supports subunit B distribution to cells. - helps epithelial cell propagation at lower levels of HSP. - has a toxic effect on epithelial cells at higher levels of HSP. - encourage the proliferation of epithelial periodontal ligament cells 	<p>(45)</p>
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CONCLUSION

The cytokines released in *A. actinomycetemcomitans* inflammation in periodontal disease are the leading cause of tissue destruction and bone resorption. New therapeutic approaches focused on *A. actinomycetemcomitans* virulence factors are expected to become part of standard clinical practice. Immunotherapies and inhibitors can be useful agents for preventing colonization of high burden or for treating infection. Although much additional work is required, it is already borne out the usefulness of targeting *A. actinomycetemcomitans* virulence factors.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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